This report contains the collective views of an international group of experts on available literature. The generation of this report followed a clearly defined process: literature search>evaluation of data and writing of the draft by a specialist (s)>review of the draft by a group of scientists>peer-reviewed by an expert (s) in the said field > and finalisation.

Assessment of Toxicological Endpoints for the Registration, Evaluation and Authorisation of Chemicals, Regulation (EC) No. 1907/2006 (REACH)

MANGANESE AND ITS INORGANIC COMPOUNDS:

2. GENOTOXICITY ASPECTS

Author: Peter Jenkinson, PhD

Director, Chemical Business Unit
Contract Research Services

Harlan Laboratories Ltd
Shardlow Business Park
Shardlow
Derby, DE72 2GD

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In accordance with the requirements of Regulation (EC) No. 1907/2006 (REACH), a literature search is required in order to assess relevant information available to the registrant, in order to include where appropriate the information into the technical dossier required for registration. One of the endpoints requiring information is genotoxicity. This report is written with a view to assisting the registrant with this requirement in mind.

The literature was searched and the search strategy is outlined below:

Literature search using Datastar:

Medline (1966+), Embase (1974+) and Toxfile (1966+) in December 2001, with a further update search being performed in August 2002.

A second literature search using STN:

The databases Medline, Embase, Biosis, HCAPLUS and Toxcenter were searched from 1959 to May 2009

In addition various reference lists from publically available review documents were also assessed and additional literature deemed relevant was obtained for review.

Author: Dr Peter Jenkinson

Peer-reviewer: Robert Durward, head of genotoxicity department at Harlan Laboratories.

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Genotoxicity Data Review for Manganese - January 2009

By Peter Jenkinson, Director Chemical Business Unit.

Summary and Conclusions

Review of Existing Data

The publications reviewed in this report only relate to soluble salts of manganese and it seems that no studies have been done on insoluble forms. The data show a mixed spectrum of results for all assay types, bacterial, mammalian cell and *in vivo*. However, in general it is clear that many of the studies were performed many years ago, before certain criteria on acceptable upper limits for testing were published and agreed (Scott et al, 1991). Many of the studies that gave positive results were difficult or impossible to evaluate because they did not provide concurrent toxicity data. Such toxicity data are absolutely necessary in order to judge the validity of the mutation data and the likely absence or presence of artefactual false positive results. *In vitro* genotoxicity assays, in particular those using mammalian cells, are prone to the induction of false positive responses by extreme culture conditions. Such artefactual results may come about because of excessive toxicity, osmolarity, pH or some other non-genotoxic mechanism. In addition, indirect genotoxic activity may be induced at dose levels which are not relevant to the whole animal, e.g. the induction of active oxygen species or the interference with enzyme activity and function.

The majority of papers reviewed on the Ames assay were negative and those that were positive were either unreliable or used highly modified procedures. Thus, it can be predicted that if a bacterial assay is performed, on any of the soluble salts of manganese, according to the standard OECD 471 test guideline, then a negative result would be obtained. There are only a small number of reports of mammalian cell chromosome aberration tests and those that are positive either used excessive levels of toxicity or did not report levels of toxicity and therefore are difficult to evaluate. One report using lymphocytes was negative in all but an unusual exposure group and even then the data were weak. It is considered likely that a negative result would be obtained in a chromosome aberration test performed to the current OECD 473 guideline using acceptable levels of toxicity, particularly if human lymphocyte cells were used because these are less prone to artefactual positive effects. There are only two reports of mammalian cell gene mutation

assays but both are positive. However, one is reported in abstract form only and cannot be evaluated whilst the other is robust but performed many years ago. The toxicity levels achieved in the second study were excessive by today's standards but are difficult to compare with those of modern methods. A mammalian cell gene mutation assay such as the mouse lymphoma test (OECD 478) may give a negative, equivocal or positive result; it is not possible to give a reliable prediction based on the available information. Mixed results have been reported in *in vivo* studies but many of the reports are poorly reported. During routine testing the vast majority of *in vitro* positive substances are negative when tested in an *in vivo* test. Consequently, an *in vivo* test on a manganese salt, such as the micronucleus assay in the mouse, is predicted to be negative. In conclusion, the available data suggest that manganese may induce mutagenic effects in genotoxicity test systems, but only when extreme concentrations are used that exceed the upper threshold levels recommended by the latest test guidelines.

With regards to the potential genotoxic potential of the oxides, carbonates and sulphides of manganese and of manganese itself and the possibility of read-across from the data for the soluble salts of manganese, the data for the soluble salts are too insubstantial and variable to be used for a confident read-across argument. However, if good quality, modern study reports were available then a read-across argument should be possible. If one accepts that manganese may show mutagenic activity under certain extreme conditions, then one potential mode of action may be presumed to be related to the presence and potential DNA reactivity of the Mn^{2+} ion (DeMeo et al, 1991). Consequently, the ability of insoluble salts of manganese to solubilise and release significant amounts of Mn^{2+} would determine its genotoxic potential. If the results of a modern dataset on a soluble manganese salt such as $MnCl_2$ indicated no potential for genotoxicity, then it may be predicted that the same result would be obtained for the insoluble salts of manganese and for manganese itself because exposure to these substances would result in much lower concentrations of Mn^{2+}. The same argument would also follow if the reported positive effects are caused by an indirect mechanism because they would still rely on adequate intra-cellular concentrations being achieved. The testing of insoluble salts of manganese, and manganese itself, is considered to be impractical.

The alternative potential mode of action is that Mn^{2+} ions may produce reactive oxygen species (ROS), particularly at high concentrations. If this was so then this mechanism would also be reliant on the presence of a sufficient concentration of

ions, which would be much less likely for the insoluble salts of manganese and manganese itself than for soluble forms.

The papers reviewed in this report are generally more than 20 years old, in many cases they are more than 30 years old. Consequently, very few of the studies they report have been performed in laboratories operating to OECD good laboratory practice (GLP) standards. Furthermore, many of the experiments are poorly reported and often lack the appropriate controls and rigour that one may expect to see in a modern report, particularly one done for regulatory purposes. In many cases the source, purity and impurity profile of the test materials are not given. Consequently, if one was to perform a Klimisch rating on these papers then the majority would score either a Klimisch 3 or 4 rating. Therefore, the validity of both positive and negative reports must be viewed with caution.

Recommendations for Further Testing

Essentially none of the *in vitro* or *in vivo* studies reviewed in this report meet the requirements of the relevant OECD test guideline. Some studies, such as those reported by Mortelmans et al (1986), Oberly et al (1982), and Lima et al (2008), come close to meeting the required standard but in each case they fall short, either because the standards have changed in the intervening years or because they have used non-standard modifications to the procedures. Of the four *in vivo* studies reviewed here three of them are substandard and/or highly questionable, whilst the fourth study could not be obtained in full. The first three are presented by their authors as showing positive results whilst the fourth study is negative.

The recommendation of this review is that new *in vitro* assays should be performed on one of the Manganese salts, according to the latest standards. It is recommended to perform a bacterial mutagenicity assay using five strains of bacteria (TA100, TA1535, TA98, TA1537 and TA102), both with and without metabolic activation and with and without pre-incubation. The maximum dose concentration should be 5 mg/plate. The pre-incubation assay using TA102 is expected to be the condition most likely to generate positive results if any of the existing studies are correct. Also a chromosome aberration test using human lymphocytes exposed using a 4-hour pulse exposure with and without S9 and a 24 hour continuous exposure is required. The maximum dose level should be 10 mM, 5 mg/ml or the dose level that induces approximately 50% reduction in mitotic index. Finally, a gene mutation assay is needed using mouse lymphoma cells in

the microtitre method and the same exposure conditions as the chromosome aberration test. However, in this case the level of toxicity should be approximately 80% inhibition of the relative total growth.

The results of these studies will be extremely useful to both judge the value of the existing studies and to determine whether or not further *in vivo* tests should be performed. If the new studies give clear negative results then it should be possible to avoid further tests using vertebrates. The choice of which salt to test is open, although in principle it seems that it should make little difference. The majority of previous studies have used $MnCl_2$ and it makes some sense to use this salt in any new studies.

Recommendations for Classification and Labelling

For mutagenicity there is **no** requirement for classification on the basis of positive *in vitro* results alone. Only positive results in an *in vivo* study will trigger the appropriate GHS Category 2 or 1b germ cell mutagen classification. In unusual circumstances a structural similarity to a known mutagen in mammals will be used to classify, even if in vivo studies on the substance itself are negative. However, this should not apply in the case of manganese.

The options for classification are:

1. Accept all the published studies at face value, in which case manganese and its salts would need to be classified as Category 2 mutagens on the overall weight of evidence.
2. Accept the weight of evidence of the *in vitro* data that manganese salts are positive, because of the relatively large number of studies reported as such. Reject the *in vivo* data on the basis that the positive reports are unreliable. In this case classification is not considered to be appropriate but new *in vivo* studies will be required to confirm or deny the *in vitro* data. In this case it is recommended to perform a micronucleus test in mouse bone marrow tissue using the intra-peritoneal route to maximise exposure and in order to confirm or deny the results of Guojun et al (2001). If positive then classification as a Category 2 germ cell mutagen will be appropriate and no further tests should be required. However, if there is evidence that manganese can reach germ cells then a classification as Category 1b will be appropriate. If the micronucleus test is negative then a second in vivo test should be considered. In this case a rat Comet assay is recommended using the oral route with examination of the stomach, liver and germ cell tissues (the latter to be confirmed). If this test is negative then no further tests are required

and manganese need not be classified. If the test is positive then classification is required as Category 2 or 1b mutagen.

3. Reject all the existing data as being unreliable. Complete the three in vitro tests described above and if negative results are obtained then no classification is required and no further testing is needed. If any positive results are obtained then it is necessary to determine which *in vivo* test is the most appropriate to perform first. If the primary effects are clastogenic (increases in numbers of cells with chromosome aberrations and/or increases in the frequency of small colonies in the MLA) then the micronucleus test is the most appropriate. If gene mutation effects predominate (Ames test positive and/or large colonies in the MLA) then the Comet assay should be performed.

The recommendation is to follow option 3 because this is the option that is least likely to result in the need for studies to be performed in vertebrate animals and is therefore the most ethical approach.

Summary of available Data

Bacterial and Yeast Assays

De Meo et al (1991). In this report various solutions or reaction mixtures of $KMnO_4$ in acidic or alkaline conditions were tested in both the standard plate incorporation method of the Ames test, and a micro pre-incubation modification. Potassium permanganate mixed with sulphuric acid gives a strongly oxidising mixture used to neutralise or decontaminate various mutagens and carcinogens in a laboratory or clinical environment. In this complicated study, potassium permanganate was dissolved in acetone, ethanol or water and mixed with sulphuric acid for 3 or 24 hours. Reactions were stopped by the addition of ascorbic acid and the mixtures neutralised by the addition of NaOH. The data are presented as volumes of the reaction mixtures and the concentration of manganese could not easily be confirmed. Some of the samples were also tested in the single cell gel electrophoresis assay, see below. In the standard assay, no activity could be detected but in the modified method, activity was seen in strains TA100 and TA102; the biggest response was seen in TA102, which is a strain designed to detect oxidative mutagens. All activity was abolished by the presence of a rat liver metabolic activation system (S9). $MnCl_2$ and $MnSO_4$ were also shown to be mutagenic to TA102 using the pre-incubation modification method but not in the standard assay format. Both salts gave similar results over a narrow dose range of 120 to 300 µg/plate. The authors conclude that Mn^{2+} was the mutagenic species responsible for the effects seen in this study. The authors support this conclusion by the observation that no mutagenic activity is seen when the reaction takes place under alkaline conditions, under which no Mn^{2+} ions are produced. This is possible but the alternative explanation of reactive oxygen species generated during the reaction process cannot be excluded. Furthermore, the inhibition of the mutagenic response by S9 is perhaps more easily explained by nullification of reactive oxygen species than by the deactivation of Mn^{2+}.

Kanematsu N (1980). Three salts of manganese ($MnCl_2$, $Mn(NO_3)_2$ and $Mn(CH_3COO)_2$ were dissolved in distilled water and shown to be not mutagenic in the rec assay in *Bacillus subtilis*. A total of 77 different metal salts gave negative results and for these the concentrations tested were not provided but they were reported to be tested up to a toxic concentration. This is a non-standard assay that uses a qualitative procedure to highlight a difference in toxicity of a chemical to two

different strains of bacteria. Any differences are presumed to be due to mutagenic activity because one of the strains is deficient in a DNA repair system. as a result of mutagenic activity. The results of tests on many different metals and their salts were reported in this paper and those that gave positive results were followed by tests using *Salmonella* or *E.coli,* although the correlation between the two systems was poor. Whilst it seems that the methods were applied in a rigorous way the test system is no longer in common use and is not considered to be a reliable indicator of mutagenicity.

Marzin DR (1985). In this study the standard plate incorporation test method was used and $MnSO_4$ and $KMnSO_4$ were tested up to the limit of solubility (1000 nM/plate) and shown to be non-mutagenic to strain TA102. A total of sixteen metal salts were tested and only two salts of chromium gave positive results. The procedures used included appropriate controls (water as the vehicle control and mitomycin C as the positive control) and five or six dose levels of the two manganese salts, in the range of 3 to 1000 nM/plate. This gives a high level of confidence in the conclusion.

Mortelmans et al. (1986). Two laboratories evaluated $MnSO_4$ in a blind test using the pre-incubation modification of the Ames test using the standard strains of bacteria (TA100, TA1535, TA98 and TA1537). No evidence of mutagenicity was detected. $MnSO_4$ was dissolved in water and tested using a range of five dose levels between 100 and 10,000 µg/plate. This paper reports the results of tests on 270 chemicals and was the second in a series of papers on the validation of the Ames test by the Stamford Research Institute and is an important early paper on the *Salmonella* assay. The methods used were the state of the art at the time and were performed within two of the three GLP laboratories that collaborated in this NTP-funded program. The data are consequently considered to be highly reliable. However, because the study was performed in the mid-eighties, strain TA102 was not included because it had not been developed by then.

Nishioka H (1975). In this short communication the results of five salts of manganese ($MnCl_2$, $Mn(NO_3)_2$, $MnSO_4$, $Mn(CH_3COO)_2$ and $KMnO_4$) were reported. The salts were dissolved in water at a concentration of 0.05M and the solutions were evaluated qualitatively in the rec assay using Bacillus subtillis by adding 50 µl to a filter paper disc placed in the centre of an agar plate. The plates were seeded with one of the two bacterial strains used in the assay; one of the strains is deficient

in DNA repair. The solutions diffuse through the agar to give a concentration gradient which is highest at the centre. Any difference in the size of the zone of toxicity surrounding the disc is presumed to be due to the mutagenic activity of the material with the larger zone in the strain that is deficient in DNA repair activity. Contrary to Kanematsu et al (1980), all of the salts gave weak positive results except for $KMnSO_4$, which was negative. The report indicates that each of the 56 metal salts observed was tested three times and the average result for each was reported. However, there was no information provided about any variation of results between replicate tests and no results were given for control materials. The relevance of these results to mutagenicity is not clear and contradicts those of the later study by Kanematsu.

Olivier P (1987). The SOS chromotest was used to examine the mutagenic potential of a series of metal salts including $MnCl_2$, $MnSO_4$ and $KMnO_4$. The assay detects DNA damage by the activation of DNA repair enzymes; all three salts of manganese were negative. A total of 48 inorganic metal compounds were tested and only two salts of chromium and one of tin showed any mutagenic activity. All the compounds were tested to their limit of toxicity using at least five dose levels in each of three replicate tests. The salts were formulated in 'L medium' at concentrations in the range of 0.3 to 100 nM/ml for $KMnO_4$ and 0.3 to 30 nM/ml for the other two salts. Volumes of 0.1 ml were added to the test vials but the methods are not reported in sufficient detail to calculate the final exposure concentrations. However, the maximum concentrations were reported as toxic so it is apparent that maximal dose levels were included in the test. It is considered that the results deserve a moderate to high level of confidence.

Parry JM (1977). Strains of Yeast were used to detect the mutagenicity of $MnSO_4$ and negative results were obtained. Eight dose levels between 1 and 500 µg/ml were used and there was some evidence of toxicity at the upper three dose levels, indicating that an appropriate dose range had been used. The method used is a non-standard study and the data are not considered to be of much value in the overall evaluation of manganese mutagenicity.

Singh I (1983). Singh used one of the same strains as Parry (1977) but achieved a positive result for $MnSO_4$ for both mutation and gene mutation. Curiously, Singh does not quote the Parry publication and therefore does not address the contradictory results. Singh diluted the manganese salt in water at 0.1 M and

exposed the cells by adding the solution to a well cut in the agar plate, thereby allowing the salt to diffuse into the agar. Supposed mutant colonies formed in a ring at the edge of the zone of toxicity. However, the author provides very little information about the methods that he used, whether or not he repeated his tests to demonstrate reproducibility, and did not include negative or positive control materials to show the functionality of his test system. On this basis the results of this paper are considered to be potentially unreliable.

Wong (1988). The chloride salts of ten 'heavy' metals, including manganese, were investigated in four strains of *Salmonella typhimurium*. A positive result was reported for $MnCl_2$ in strain TA1537 in the absence of S9 only. However, only derived data are presented and the actual plate counts are not given. Furthermore, the spontaneous number of revertants, reported by the author in his experiments with TA1537, was given as 60, a number that is 4 to 20 times the normal range quoted by Bruce Ames. It seems likely therefore, that the culture of TA1537 used by Wong was contaminated and consequently the data must be considered to be unsound. The author gives no details of the number of concentrations used between the 50 and 90% toxicity levels that he identified in his preliminary experiments. However, if all the dose levels used were at least 50% toxic then it is almost certain that he ran the risk of observing toxicity-related artefacts. Therefore it is impossible to evaluate the validity of the conclusion.

Zakour (1984). It is not clear whether this study should be included in this section, because it is a bacterial assay using *E.coli*, or in the non-standard assay section, because it is certainly a non-standard assay which has never been used in any regulatory testing strategy. The assay detects mutants of the *LacI* gene and the experiments showed a modest increase in the frequency of mutants when the cells were grown in the presence of $MnCl_2$ at concentrations of 5, 10, 25 and 50 µM; the highest dose level being toxic. The data were not highly reproducible in that there was a large standard deviation seen between the results of a large number of cultures, whereas a small SD was typical for control cultures. Furthermore, the types of mutations seen were typical of control cultures and the dose-response relationship was poor. The toxicity of the concentrations used was reported to be the maximum that could be tolerated and still achieve sufficient growth for analysis. It is not possible to fully evaluate the significance of these results because the assay in not validated nor in common use.

Mammalian Cell Assays

Andersen O (1983). Anderson reports increases in sister chromatid exchange (SCE) frequency in two types of mammalian cell lines (P388D1 and HL) after exposure to $MnSO_4$. However, the increases are modest (maximum 45%) and the actual numerical data are not given. Furthermore, there are no concurrent data for the level of cytotoxicity induced by the exposure levels used. Indeed, there is some confusion as to what concentrations were used. In Table 2 of the report the 'approximate' maximal concentrations allowing SCE determination was given as 10^{-5} M for both cell lines. However, in Table 9 the data are presented as below:

Cell Type	M	Increase in SCE compared to control, %	*p*
P388D1	10^{-4}	45	<0.005
P388D1	10^{-5}	39	<0.0125
P388D1	10^{-6}	30	<0.05
HL	10^{-7}	13	NS
HL	10^{-6}	9	NS

There is a discrepancy between the maximum dose concentration given in table 2 and the one given in table 9 for the P388D1 cell line by an order of magnitude. Furthermore, the concentrations used with the HL cell line are not given in the correct sequence so it is not possible to know whether the concentrations are given correctly, incorrectly or just in the wrong order.

The SCE assay is acutely prone to weak false positive results because toxicity can induce cell-cycle delay, which is in itself responsible for modest increases in SCE frequency even in the absence of mutagenicity. Whilst it is not possible to evaluate the validity or relevance of these results the discrepancies in the paper give rise to a high level of caution about their reliability.

De Meo et al (1991). The authors present single cell gel electrophoresis (Comet assay) data for human lymphocytes exposed to a reaction mixture of $KMnO_4$ designed to produce strong oxidising conditions (see the comments above in the section on bacterial assays). They also report positive results with $MnCl_2$ at concentrations of 1.5, 3 and 4.5 mM, although the dose response relationship was weak and the standard deviations for each dose level were very high. The authors themselves accept that it was not possible to do a quantitative evaluation of these data. The dose levels also seem to be extremely high, being very close to the maximum recommended concentration of 10 mM. The most likely explanation for this response is the production of reactive oxygen species and it is not clear that manganese was responsible for the DNA damage monitored by this assay although the authors conclude that Mn^{2+} was responsible for the DNA damage that they observed. They base this conclusion on the fact that under acidic conditions MnO^{4-} gives rise to Mn^{2+} ions but under alkaline conditions manganate ions are produced (MnO_4^{2-}). In their experiments the latter samples were negative but the former samples were positive. It should also be noted that this is an early paper in the development of this procedure (first described in 1988), and the authors themselves acknowledge that there are many questions on the test method and it's reproducibility that need to be answered. Even now there is no OECD standard method, although a multinational programme in under way to achieve consensus on test methods in order to prepare an international standard method.

Galloway (1987). Manganese (II) sulphate monohydrate was investigated for its potential to induce SCE and chromosome aberrations in CHO cells. Positive results were reported for SCE, both with and without S9, whilst positive results for aberrations were only observed in the absence of S9. In the case of the SCE data, in one experiment the exposures to $MnSO_4$ were not properly controlled because a longer exposure time was used for the test treatment than the control. The longer time was used to allow sufficient cells to accumulate for full evaluation; the implication is that the treatment was too toxic. In fact there are no concurrent toxicity data provided in the report and therefore it is not possible to determine whether the dose levels used were within an acceptable range of toxicity. A similar situation exists for the chromosome aberration data in that no concurrent toxicity data are provided. However, the report states that additional culture time in the presence of Colcemid (the agent used to accumulate metaphase cells) was required, which suggests that the level of toxicity achieved in this study was excessive according to modern standards. In conclusion, the data indicate that, for

both studies, the dose levels that were used were in excess of the levels that are considered appropriate to avoid toxicity-induced artefactual effects.

Lima PD (2008). This modern study applied a protocol using human lymphocyte cells to study the clastogenic and DNA damaging effects of $MnCl_2 \cdot 4H_2O$ used at concentrations of 15, 20 and 25 µM. The upper concentration was the maximum that could be used before toxic effects prevented the production of sufficient cells in metaphase for analysis, i.e. the toxic limit for the assay. The protocol design had some similarity to the standard OECD 473 test guideline in that both continuous and pulse exposure conditions were used. However, no tests were performed in the presence of S9. Under these exposure conditions negative results were obtained and in all cases the level of toxicity achieved exceeded the 50% cell growth inhibition level required by the OECD guideline. In some cases levels of toxicity were excessive but even so no significant induction of cells with aberrations was observed. Positive results were seen using a very short exposure time of 3 hours at the end of the 72 hour culture period. Very little toxicity was observed in this case, although the reduction in mitotic indices was statistically significant at the two highest concentrations. The majority of aberrations that were observed were chromatid gaps and it is known that many of these are not real aberrations. Therefore, the relevance of these findings is not clear. The data suggest that a properly conducted OECD 473 chromosome aberration study would yield negative results.

Oberly et al (1982). In this study the L5178Y mouse lymphoma assay was used to evaluate the gene mutation activity of various metal salts. The method used is comparable to the standard OECD 478 test guideline except that the method of estimating toxicity and plating for survival and mutation has now been superseded. $MnCl_2$ was negative in the presence of metabolic activation (S9) but clearly positive in the absence of S9 and the results showed a dose-related response at dose levels giving 50% or greater toxicity (a response was seen at a lower level of toxicity but this was within the solvent control range for the study). However, the method of estimating toxicity used in this study would be expected to underestimate toxicity compared to the accepted latest method. Over the last 15 years it has been shown that this assay can easily provide false positive results if excessive toxicity is achieved. The agar method used in this study is particularly prone to this problem because cells affected by toxicity grow more slowly than normal cells, which is exacerbated by the agar, which also slows growth and reduces the ultimate size of the colony. In such cases it is easy to underestimate the number of surviving

colonies, which then affects the accuracy of the calculation of mutation frequency. The microtitre plate method is more commonly used today and is less prone to such problems. Consequently it is not possible to predict whether a regulatory study performed to a modern protocol would yield a positive response or not because the response seen by in this study may or may not have been an artefact of excessive toxicity.

Umeda (1979). FM3A cells were used to investigate the clastogenicity of a number of metal salts including $MnCl_2$ (0.1, 0.32, 0.64 and 1.0 mM) and $KMnO_4$ (0.64, 1.0, 2.0 and 3.2 mM). $MnCl_2$ was reported as negative at concentrations that included a high level of toxicity, whereas $KMnO_4$ was reported as positive. In the case of $KMnO_4$ the concentrations used were very high and the responses seen quite weak, in that many of the aberrations seen were of the 'gap' type, which may or may not be true aberrations. Furthermore, the response was observed in a very narrow concentration range, presumably at or near the toxic limit of the assay system. No concurrent toxicity data are provided in the report and so it is not possible to properly evaluate the validity of the data. However, it is likely that the effects seen may have been an artefact of excessive toxicity.

Zelikoff JT (1986). This is an abstract report only of a gene mutation assay using V79 cells. The author reports positive results for soluble salts of manganese but there are no data given in the report and it is impossible to evaluate the validity of the conclusion

In vivo Assays

Dikshith et al (1978). In this study rats were orally dosed daily with 50 µg/kg for 180 days and bone marrow and spermatogonial cells were analysed for chromosome aberrations. No increases in clastogenic effects were observed. No indication of the toxicity of the dose exposure was indicated other than the fact that no animals died in the study, therefore the justification for the dose level used was not clear, but it would seem to be a very low dose level or perhaps a miss-print. The acute oral LD50 for MnCl2 in mice is ~450 mg/kg, ie ~9000x the dose used in this study. Long-term studies have reported no effects with dose levels as high as 50 mg/kg/day. So, either Dikshith has used a dose ~1000x less than he could have done or the units are mis-printed as µg instead of mg. If the units are correct then

the data are probably worthless. If they were mg/kg, then they are encouraging but we cannot use them with confidence.

Guojun et al. (2001). In this relatively recent study, mice were dosed with 25, 50 or 100 mg/kg of $MnCl_2$ via the intraperitoneal route. Breast bone marrow cells were examined for the frequency of micronuclei, which provide evidence of clastogenic (chromosome structural damage) or aneuploidy (change in chromosome number) effects. The data shown indicate a clear dose-related and highly significant increase in the frequency of micronuclei. However, the study is reported in Chinese with only a summary in English and so it is not possible to fully understand the method used. The data are perhaps a little suspicious in that, in the experience of the reviewer, the dose response is impressive and much more positive than one might expect for a chemical that has an equivocal mutagenic profile. Furthermore, the i.p. LD50 of $MnCl_2$ in the mouse is reported as being only 121 mg/kg, which is very close to the maximum dose level used in this study. It is unusual to sample breast bone marrow when the standard tissue is the femur bone marrow, which is much easier to access. Furthermore, the ratio of polychromatic to normochromatic erythrocytes was not reported. This ratio is a good indicator of toxicity to the bone marrow and may be used to validate the dose level selection. The author provides no evidence of historical data for the strain of mouse or tissue used. In conclusion it is not possible to evaluate the validity of this study.

Joardar et al (1990). In this study mice were dosed orally with either $MnSO_4$ (at 102.5, 205 or 610 mg/kg) or $KMnO_4$ (at 65, 130 and 380 mg/kg) and examined for chromosome aberrations after 7, 14 or 21 days of dosing and for micronuclei in bone marrow cells after 2 doses 24 hours apart. Mice were also dosed for 5 days and evaluated for abnormalities in sperm 30 days later. The maximum dose level was 1/5 of the LD50, the mid-dose 1/15 and the low dose 1/30. The result for all three end-points indicates positive genotoxic effects. However, there is a lack of clarity on the scoring system, in particular for chromosome damage that raises some doubt about the technical expertise of the authors and their understanding of the test systems used. Furthermore, a dose-related increase in effect was seen but not with increased exposure time. This was explained by a possible saturation of sites for binding of the Mn^{2+} anion. However, if this was the case then it is not clear why saturation would also not occur as the dose level was increased. This inconsistency raises some doubt as to the validity of the data.

Newell, G. (1974). This study reported the investigation of the genotoxic effects of manganese sulphate in male rats dosed orally for 1 to 5 days (dose-levels were not reported). Negative results were given for the induction of heritable translocations and dominant lethality. In this case the full report was not available for review but was described in an NTP Technical Report No. 428. However, the report has good credibility because it was done on behalf of the US FDA.

Non-Standard Assays

Baranowska H (1977). The authors report positive results for mutagenicity in yeast. However, the dose levels used in the tests were very high (6 or 8 mM), and close to the maximum recommended level of 10 mM for in vitro assays. No dose response was shown for the effects seen so it is difficult to know if they were subject to a threshold. It was observed that no cell replication took place during the exposure periods which suggests that the exposures were extremely toxic and that the responses may have been artefactual. It is considered that these data have no value for the evaluation of manganese mutagenicity.

Casto et al (1979). The assay system used in this report was designed to determine any enhancement of cell transformation induced by viral infection. The mechanisms that lead to cell transformation are multi-factorial and include non-mutagenic actions, therefore these data are of no use in the evaluation of the mutagenicity of manganese.

Snyder RD (1973). A fibroblast cell line was used to detect and investigate the nature of DNA strand breaks induced by exposure to a series of metal salts including $MnCl_2$. The data clearly show that $MnCl_2$ does induce DNA damage but only at very high concentrations of 10 mM and above but not at 5mM. 10 mM is the maximum concentration that should be used in order to avoid artefacts of hyper osmolarity. However, the effects of $MnCl_2$ were inhibited by the presence of protein (Foetal Bovine Serum) and the DNA damage was shown to be linked to the generation of oxygen radicals.

Valencia (1985). This study used *Drosophila melanogaster* to investigate the potential genotoxic effects of $MnSO_4$ on the germ cells of male flies. The studies were performed by three US Universities as part of the NTP trial on genotoxicity assays begun in the early 1980's. No significant effects were observed and the conclusion of the study was negative. This assay is not a component of any regulatory test battery or scheme and its validity and usefulness have never been fully accepted. However, its proponents argue strongly in its favour and generally it scores well in comparisons of predictivity. However, in this case the study is probably of little use for the purposes of compliance with REACH.

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Peter Jenkinson 05 MAR 2009